THE MECHANISM OF ACTION OF ENDOTOXIN

31 October 1962 to 31 October 1963

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ABSTRACT

1. **Preparing Institution:** University of Pittsburgh School of Medicine
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2. **Title of Report:** The Mechanism of Action of Endotoxin

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Evidence from this laboratory and others indicates that endotoxin shock is a form of anaphylactic shock mediated through natural antibody and characterized by complement-fixation in vivo; that resistance to endotoxin shock can be increased by the acquisition of further antibody; and that intestinal bacteria may influence susceptibility to endotoxin by their effect on the production of both natural and acquired antibody. These immunologic factors in the production of endotoxin shock were investigated further by the following studies:

A. A Systematic Analysis of the Reaction Between Endotoxin and Complement. The inactivation of complement by an endotoxin varied with the bacterial species from which the endotoxin was derived and with the individual serum examined. Considerable differences in the depression of complement levels were observed among different endotoxins and different subjects. Each individual, however, exhibited its own unique pattern of complement inactivation with the battery of endotoxins employed and the susceptibility to complement inactivation was stable from week to week. A good correlation was found between antibody (hemagglutinins) to endotoxin and complement inactivation by endotoxin; but lethality of endotoxin could not be related to its anticomplementary action in vitro. In experimental bacteremic shock from E. coli, however, the same drop in C' levels in vivo were observed as in animals given lethal doses of endotoxin. In sublethal joint infections with E. coli the circulating levels of endotoxin produced transient falls in C' levels in half the animals and eventual elevations in circulating complement in all. These results strengthen the thesis that complement is involved in reactions with endotoxin in vivo and in vitro through a reaction with natural antibody; but the role of complement in mediating the lethal reaction in endotoxin shock requires further study to explain why C' depression in vitro and lethality of endotoxin could not be correlated with each other.

B. Immunization Against Bacteremic Shock by Feeding Viable E. coli. Feeding E. coli O:113 for 3 weeks stimulated antibody to the homologous endotoxin and protected animals with complete neutropenia against bacteremia arising from thrombophlebitis of pelvic veins. Antibody to endotoxin appeared to reduce the invasiveness of E. coli.

C. The Influence of Immunologic Paralysis Induced by Mitomycin C On Susceptibility to Endotoxin.
The DNA antagonist, Mitomycin C, which inhibited antibody formation to endotoxin, lowered resistance to infection by Gram negative bacteria and to the lethal effects of their endotoxin.

D. A Comparison of the Toxic and Antigenic Properties of Endotoxin Purified by Density Gradient Zone Ultracentrifugation.
The major toxic and antigenic activities were found together in purified fractions of endotoxin that localized in the dense portions of the gradient, while the bulk of the material was least dense and showed little biologic activity. These studies further illustrated the parallelism of the toxic and antigenic properties of endotoxin and imply that a single molecular configuration is responsible for both properties. This new evidence that toxicity and antigenicity go hand in hand is consistent with the view that endotoxic shock is a form of anaphylactic shock.

Copies of this report are filed with the Armed Services Technical Information Agency, Document Service Center, Knott Building, Dayton 2, Ohio, and may be obtained from that agency by qualified investigators working under Government Contract.

INTRODUCTION

Evidence from this laboratory and others indicates that endotoxin shock is a form of anaphylactic shock mediated through natural antibody and characterized by complement-fixation *in vivo*; that resistance to endotoxin shock can be increased by the acquisition of further antibody; and that intestinal bacteria may influence susceptibility to endotoxin by their effect on the production of both natural and acquired antibody. These immunologic factors in the production of endotoxin shock were investigated further by the following studies:

A. A systematic analysis of the reaction between endotoxin and complement.

B. Immunization against bacteremic shock by feeding viable *E. coli*.

C. The influence of immunologic paralysis induced by Mitomycin C on susceptibility to endotoxin.

D. A comparison of the toxic and antigenic properties of endotoxin purified by density gradient zone ultracentrifugation.

A. Analysis of the Reaction Between Endotoxin and Complement.

1. Comparison of the Action on Complement of Endotoxins from Different Bacterial Species.

The original studies from this laboratory, reported last year, demonstrated that lethal doses of endotoxin from E. coli, O:113 and Proteus consistently depressed serum complement levels in rabbit blood in vivo. This depression of serum complement in vivo was duplicated in vitro by incubating serum with these two endotoxins in concentrations present in the circulation blood of rabbits immediately after injection of 5.0 mg., the minimal 100% lethal dose (MLD_{100}). In order to determine the extent of this phenomenon among different endotoxins, and in different animal species, complement levels were measured in sera from rabbits, rats, and humans before and after exposure to endotoxin in vitro.

Blood was drawn from the heart of rabbits and rats and from the antecubital vein of healthy adult humans into tubes placed in an ice bath. Serum was separated by centrifugation at 4° C. and stored at -70° C. The serum was thawed just before the test at 4° C. and warmed by incubating at 37° C. for 5 minutes. The effect of endotoxin on serum

complement was determined by mixing 0.9 ml. serum with 0.1 ml. of a solution of endotoxin in saline. The complement level was compared with that in a portion of the same serum to which saline alone was added. The serum plus endotoxin, and serum plus saline were incubated for 1 hour at 37° C. in a water bath and transferred immediately thereafter to an ice bath for determination of complement levels by the method of Osler, Strauss, and Mayer (1). The endotoxins were obtained from the Difco Company, where they were extracted from the various bacterial species with trichloracetic acid by the method of Boivin. The final concentration of endotoxin in serum was .066 mg./ml. This concentration of endotoxin is equivalent to the concentrations present in the circulating blood of 3.0 Kg. rabbits immediately after injection of 5.0 mg., the MLD_{100}. Previous studies (2) with E. coli 0:113 endotoxin had shown that complement levels were consistently depressed in vivo only with consistently lethal doses.

The results are shown in the following figures and tables:

INFLUENCE OF BACTERIAL SPECIES ON COMPLEMENT INACTIVATION BY ENDOTOXIN IN 10 DIFFERENT RABBIT SERA

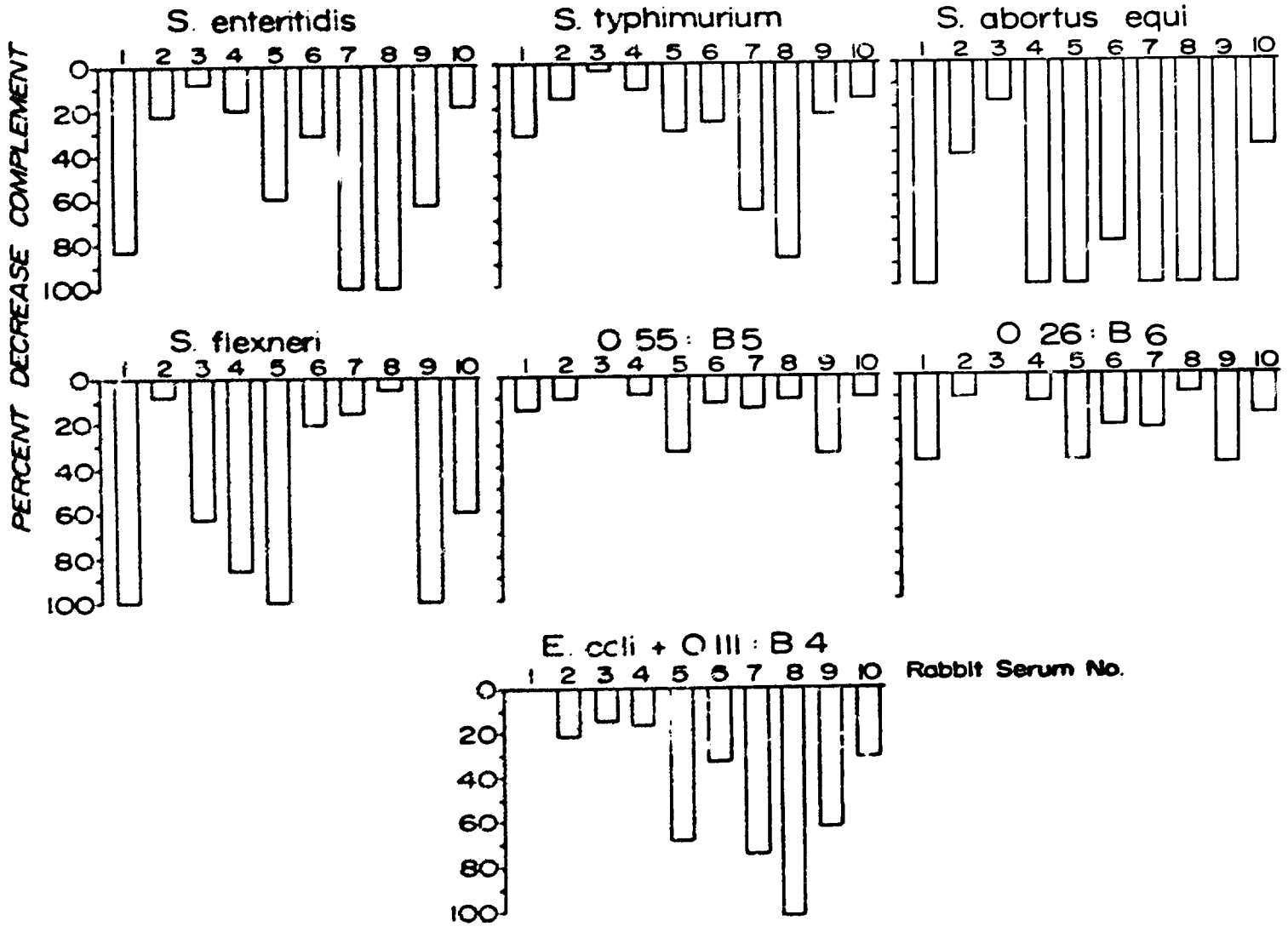


FIGURE 1

INFLUENCE OF BACTERIAL SPECIES ON COMPLEMENT INACTIVATION BY ENDOTOXIN IN 10 DIFFERENT HUMAN SERA

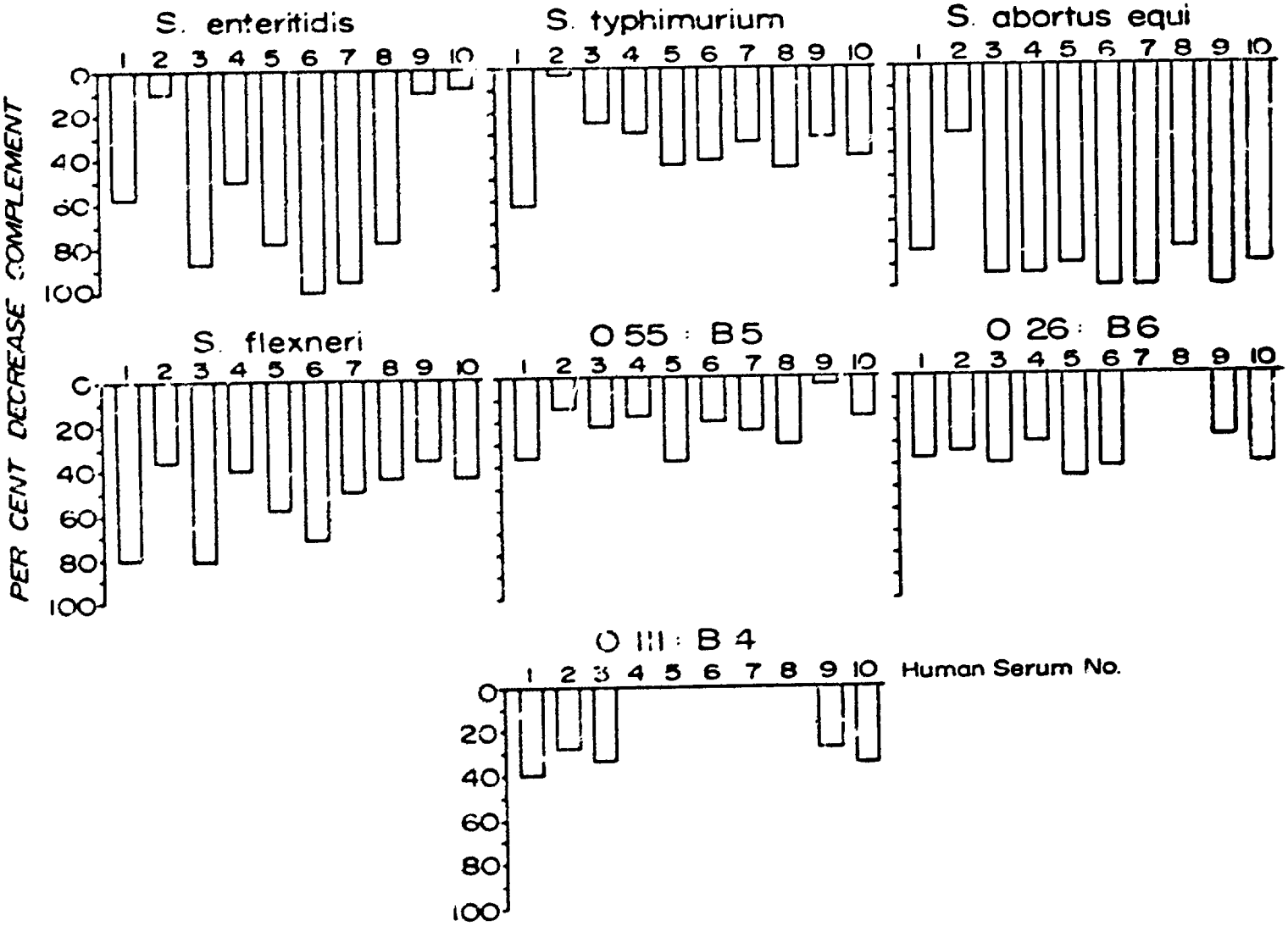


FIGURE 2

RABBIT SERA

Per Cent Fall in C' Level After 60' Incubation With Endotoxin

Serum #	Salmonella enteritidis	Salmonella typhimurium	Salmonella abortus equi	Shigella flexneri	E. coli 055:B5	E. coli 026:B6	E. coli 0111:B4
1	83.2	33.6	100.0	100	17.6	39.2	
2	23.9	16.6	42.3	9.2	11.0	11.7	22.1
3	9.6	3.7	17.4	63.3			14.7
4	21.0	12.0	100.0	86.4	9.0	13.7	16.6
5	59.6	30.9	80.0	100	34.0	39.4	69.1
6	32.1	27.4	100.0	21.7	13.2	23.6	33
7	100.0	66.7	100.0	16.7	15.6	24.4	75.6
8	100.0	87.5	100	7.9	11.2	9.9	100
9	63.9	23.8	100	100.0	37.7	41	63.9
10	18.8	16.4	37.2	60.4	10.6	18.4	32.4

ADULT HUMAN SERA

Per Cent Fall in C' Level After 60' Incubation With Endotoxin

Serum #	Salmonella enteritidis	Salmonella typhimurium	Salmonella abortus equi	Shigella flexneri	E. coli 055:B5	E. coli 026:B6	E. coli 0111:B4
1	59.3	63.1	84.5	81.2	36.5	41.3	40.1
2	8.1	3.5	32.3	37	14.2	33.6	28.5
3	87.4	35.1	93	82.4	21.9	39.3	33.5
4	49.2	30.9	94.3	39.9	18.3	28.8	30.9
5	78.4	44.2	90.3	58.9	37.9	45.8	
6	100	42.0	100.0	71.5	20.4	43.4	
7	95.8	33.7	100.0	49.4	24.2		
8	77.6	45.4	83.1	44.4	30.6		
9	10.8	32.4	100.0	35.4	2.9	28.7	25.9
10	8.7	38.3	89.6	45.7	18.2	40.3	34.8

These results show that endotoxins from different bacterial species vary greatly in their action on complement. Endotoxin from S. abortus equi is the most active in depressing complement in vitro in both human and rabbit serum, and is equally active in rat serum. The order of complement-depressing activity of different endotoxins is as follows in human and rabbit serum:

	Human	Rabbit
1.	S. abortus equi	S. abortus equi
2.	S. enteritidis and S. flexneri	S. enteritidis
3.		S. flexneri
4.	S. typhimurium	E. coli 0111:B4
5.	E. coli 0111:B4, 026:B6, 055:B5	S. typhimurium
6.		E. coli 026:B6 and 055:B5

The similarity in the order of anticomplementary activity of different endotoxins in rabbit and human serum is surprising because it is not readily explained on the basis of a reaction of endotoxin with natural antibody acquired through prior exposure (subclinical infection) to the bacillus from which the endotoxin was derived. Human beings are much more constantly exposed to E. coli than S. abortus equi, yet endotoxins from various species of E. coli exhibited far less ability to inactivate complement. It would also seem unlikely that rabbits and man would develop

the same pattern of natural antibody to endotoxins, since rabbits usually possess no E. coli in their bowel and are not exposed to the same bacteria as human beings.

These experiments also show that individual rabbits and people show a unique pattern of reaction between their serum complement and different endotoxins. These unique individual patterns are shown in Figures 1 and 2. They illustrate marked individual differences in susceptibility to an effect of endotoxin that have not been previously appreciated.

2. Comparison of the Reaction Between Different Endotoxins and Complement in Sera Obtained at Different Times from the Same Person.

In order to determine the constancy of the reaction between endotoxin and complement in the serum of individual subjects, 50 ml. of blood was drawn from two volunteers 3 times at weekly intervals and the serum stored at -70° C. as described above. After all samples had been collected they were examined simultaneously for the effect of 7 endotoxins in concentrations of .066 mg./ml. on the complement levels with the following results:

	Endotoxin	Per Cent Decrease in C' Levels After 1 Hour Incubation With Endotoxin		
		First Week	Second Week	Third Week
Subject #1	S. enteritidis	38.5	41.1	55.4
	S. typhimurium	41.5	48.8	63.7
	S. abortus equi	94.2	100.0	86.5
	S. flexneri	48.0	47.6	45.0
	E. coli 055:B5	31.7	29.1	27.2
	E. coli 026:B6	35.5	35.2	33.1
	E. coli 0111:B4	32.1	34.0	31.3
Subject #2	S. enteritidis	57.4	64.8	74.2
	S. typhimurium	35.6	38.3	37.5
	S. abortus equi	100.0	100.0	100.0
	S. flexneri	47.2	58.9	56.4
	E. coli 055:B5	35.1	26.7	26.5
	E. coli 026:B6	26.8	38.1	34.9
	0111:B4	26.8	26.3	27.0

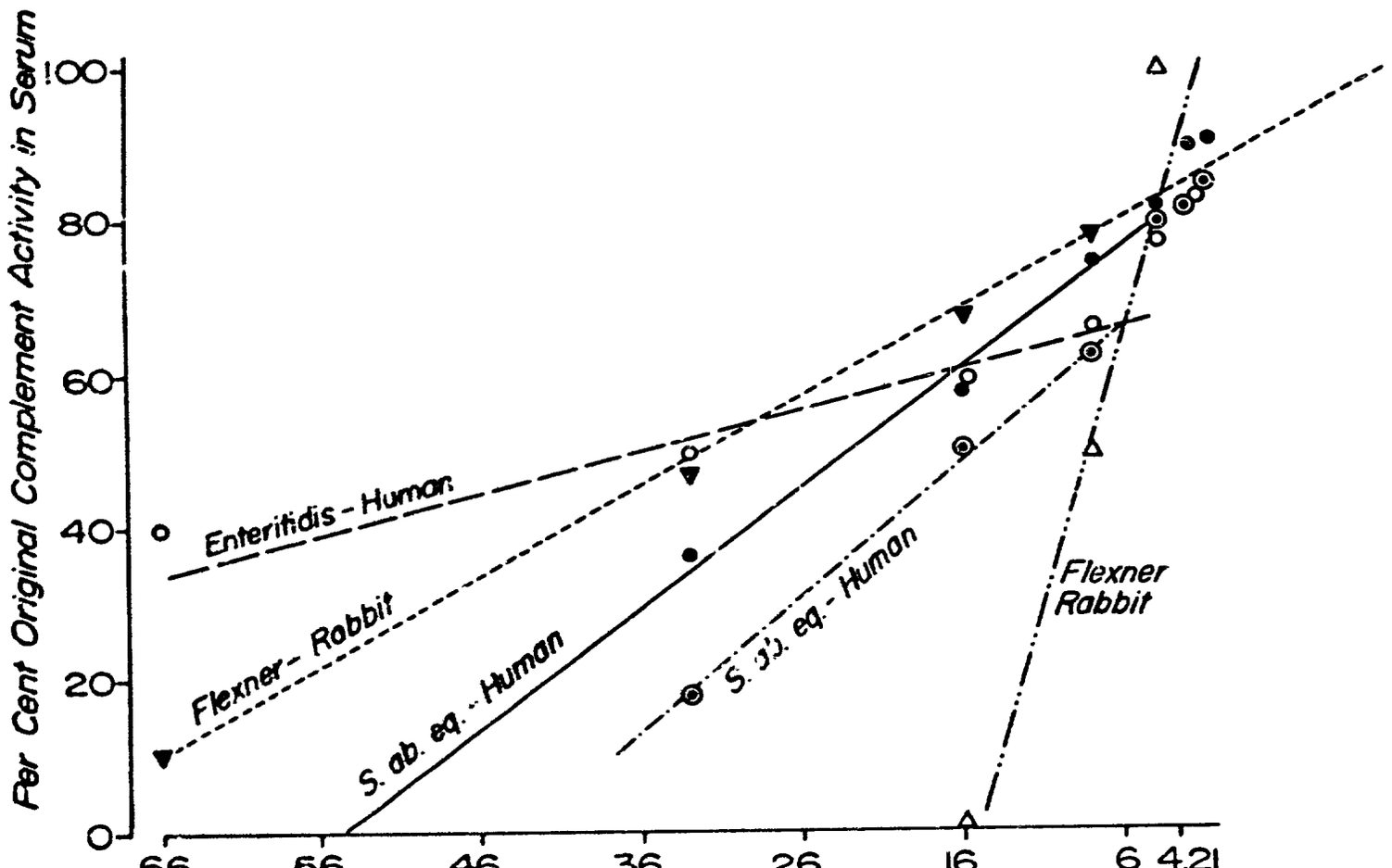
The results indicate that the pattern of individual complement depression by different endotoxins represents a stable reaction with the serum of any given individual. The complement levels in untreated serum are also stable. In Subject 1, they were 46.7, 47.7, and 49.6 units respectively during the 3 consecutive weeks; in subject 2 they were 48.1, 45.7, and 39.2 units during the 3 consecutive weeks.

3. Titration of Endotoxins for Anticomplementary Dose-Response Curve.

The reaction of complement with different doses of endotoxin was determined by measuring complement activity after incubation

FIGURE 3

INFLUENCE OF CONCENTRATION OF ENDOTOXIN ON COMPLEMENT INACTIVATION BY DIFFERENT ENDOTOXINS



The source of the endotoxin (S. enteritidis, S. flexneri, S. abortus equi) and the type of serum (human or rabbit) are indicated over each line.

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of human and rabbit serum with serial dilutions of endotoxin. The procedure was the same as that used in the preceding experiments.

The 5 sera that displayed a linear type of inactivation curve for complement are shown in Figure 3. The following summary gives the results of the titration for the 11 sera examined with either S. abortus equi, S. enteritidis, or S. flexneri.

Per Cent Decrease in C' Levels After 1 Hour Incubation With Endotoxin

Concentrations micrograms/ml.	S. abortus equi				S. enteritidis				S. flexneri		
	Human Sera		Rabbit Sera		Human Sera		Rabbit Sera		Rabbit Sera		
	1	2	1	2	1	2	1	2	1	2	3
66	100	100	39.3	91	40	59.8	18.2	34.6	90.5	38	100
33	63.6	81.7	32.5	91	40	51.4	6.8	24.7	43	38	100
16	42.5	56.6	26.5	21.5	30	42.4	11.4	18.9	32	32.8	100
8	30.4	38.6	18.8	27.8	21	34.9	10.6	15.2	22	29	50
4	17.3	21.6	13.7	19.4	12	21.9	12.1	7.4	0	0	0
2	10.5	15.3	10.3	16.0	10	14.9	10.6	8.2	0	0	0
1	7.7	13.8	8.5	11.8	10	12.4	10.6	8.2	0	0	0

The curves obtained in this experiment (Figure 3) illustrate further that the reaction between sera and endotoxin varies with either reagent, i.e., serum or endotoxin. The linear reactions have variable slopes (Figure 3) and the others are irregular in shape. An interesting feature of certain

sera (Human 1 S. enteritidis; Rabbit 2 S. flexneri) is that progressive increase in dosage does not always lead to further inactivation of complement beyond a certain fraction.

4. Relationship Between Complement Inactivation by Endotoxin, Heterophile Antibody, and Natural Antibody to Endotoxin.

In order to determine whether complement is inactivated in vitro as a result of a reaction between natural antibody and endotoxin, a comparison was made between the titer of natural antibody to endotoxin and the amount of complement depression. Because Shigella flexneri endotoxin is antigenically related to the heterophile antigen in sheep cells, heterophile antibody in normal rabbit serum was also examined for any possible role it may play in the inactivation of complement by endotoxin.

The sera of 26 rabbits were divided into 4 portions. These were examined as follows: (1) Natural antibody to S. flexneri endotoxin was determined by measuring the titer of hemagglutinins to human group O red cells sensitized with that endotoxin by the technique of Neter (3). The heterologous agglutinins for human group O red cells were first removed from

rabbit sera by absorption with group O cells. (2) Complement levels were determined after incubation for 1 hour at 37° C. with 66 micrograms S. flexneri endotoxin and compared with the levels in portions of the same sera incubated without endotoxin. (3) The heterophile titer was measured by hemagglutination of sheep red cells. The sera were inactivated for 30 minutes at 56° C. and .25 ml. was serially diluted with .25 ml. saline. Then 0.1 ml. of a 2% washed suspension of sheep cells was added to each tube and hemagglutination observed after incubation at 37° C. for 1 hour and at 4° C. overnight. (4) Inhibition of sheep cell agglutination was attempted by incubating each serum with 66 micrograms S. flexneri endotoxin for 1 hour at 37° C. before the heterophile test; and the heterophile titer then compared with that in portions of the same sera incubated similarly but without endotoxin.

The following results were obtained:

(a) Relationship between complement inactivation by endotoxin to titer of natural antibody (Hemagglutinins) to S. flexneri endotoxin is shown in the following table.

Rabbit #	% Fall in Complement After Incubation With S. flexneri	Hemagglutinin titer to Endotoxin Sensitized Red Cells
1	100	64
2	100	64
3	100	16
4	100	8
5	100	8
6	100	8
7	100	8
8	100	8
9	91	8
10	44	8
11	100	[illegible]
12	100	4
13	100	4
14	100	4
15	65	4
16	58	4
17	50	4
18	29	4
19	23	[illegible]
20	10	4
21	11	2
22	64.5	0
23	13.7	0
24	3.5	0
25	0	0
26	0	0

This table can be summarized as follows:

Antibody Titer	Number of Sera	Average Depression in C' Level After Incubation With Endotoxin	Per Cent Sera Showing a C' Fall 30%
0-2	6	16%	16
4	12	54%	60
8 and above	8	94%	87

A correlation is thus suggested between antibody titer to endotoxin (as measured by hemagglutination) and the degree of inactivation of complement by endotoxin. It is consistent with the view that complement inactivation by endotoxin results from complement fixation by natural antibody to endotoxin.

(b) Relationship between complement depression by endotoxin and heterophile antibody:

Rabbit	Reciprocal of Serum Titer of Sheep Cell Agglutinins With Endotoxin	Without Endotoxin	Depression of C' Level By Endotoxin
1	8	8	100
2	4	4	100
3	8	8	100
4	8	8	64.5
5	2	2	57.5
6	4	4	29.0
7	2	4	14
8	8	8	11.1
9	8	8	10
10	4	4	9
11	4	4	3.5
12	8	8	0

These results demonstrate that the inactivation of complement by S. flexneri was not related to reactions with heterophile antibody because it was independent of the heterophile titer of the serum; and because this endotoxin did not block sheep cell agglutination in sera that lost C' activity

after exposure to endotoxin.

5. Relationship Between In Vitro Complement Inactivation and Lethality of Endotoxin.

In previous studies we have reported that endotoxin produced a consistent fall of complement levels in vivo only with consistently lethal doses. This raised the question of how complement depression was related to the lethal effect of endotoxin — Was complement depression a causal factor, was it coincidental, or was it a result of lethal intoxication? This question was difficult to answer when changes in C' were measured in vivo because direct correlations could not be made in individual animals between complement changes and lethality in view of the act that removal of blood for C' assay after injection of endotoxin affected lethality. Instead it was necessary to measure C' levels and lethality separately and correlate the results between the two groups of animals. In order to relate changes in C' with lethality in the same animal, it was necessary to compare the fall of serum C' induced by endotoxin in vitro with lethality to endotoxin injected into rabbits after they had recovered from removal of blood for C' assay.

Blood was removed by cardiac puncture from 52 rabbits. Each serum was incubated with 66 micrograms of Shigella flexneri endotoxin per ml. for 1 hour at 37° C. and the fall in complement level determined by the method described in A-1. The lethality in rabbits whose sera showed a sharp fall in C' level was then compared with that in rabbits resistant to the anticomplementary effects of S. flexneri endotoxin. The following results were obtained:

	Marked Fall in C' in vitro			Little, if any, Fall in C' in vitro		
Dose Endotoxin (mg)	Rabbit	% Fall in C'	Survival Time (Hr. After Endotoxin)	Rabbit	% Fall in C'	Survival Time (Hr. After Endotoxin)
5	1	100	2	A	0	8
	2	100	8	B	0	9
	3	100	8	C	0	9
	4	100	8	D	0	12
2.5	5	100	3	E	6.3	20
	6	100	8	F	6.5	20
1.25	7	92.3	20	G	8.7	6
	8	93.2	5	H	20.4	7
	9	84.1	12	I	8.9	12
	10	90.7	20	J	6.8	8
	11	94.0	20	K	17.6	12
.625	12	100	Survived	L	10.8	Survived
	13	100	Survived	M	21.8	24
	14	100	72	N	0	12
	15	100	Survived	O	8	Survived
	16	100	Survived	P	29	Survived
	17	100	72	Q	10	16
	18	100	12	R	9	24
	19	100	72	S	14	72
.312	20	100	Survived	T	11.1	Survived
	21	100	Survived	U	0	12
	22	100	Survived	V	13.7	Survived
	23	100	Survived	W	3.5	Survived
	24	100	Survived	X	11	Survived
	25	100	Survived	Y	8	Survived
	26	100	18	Z	17	Survived
	27	100	18	AA	13	Survived

The LD_{50} of S. flexneri endotoxin in the two groups were:

Group I — Marked fall in C' = .525 mg. LD_{50}

Group II — Little, if any fall in C' = .744 mg. LD_{50}

These results show that complement inactivation in vitro was not related to lethality of endotoxin.

6. Complement Levels During Infection by Gram Negative Bacteria That Contain Endotoxin.

In order to determine whether circulating endotoxin affected complement levels during infection by Gram negative bacteria, rabbits were subjected to two types of infection with E. coli: (a) Lethal bacteremic shock. In this type of infection, in which lethal doses of endotoxin are held responsible for the fatal bacteremia, C' levels would be expected to fall as they do in rabbits given lethal doses of purified endotoxin. (b) Septic arthritis of the knee due to E. coli. In this type of infection sublethal doses of endotoxin are present in the circulation and would not be expected to reduce C' levels consistently.

(a) Lethal Bacteremic Shock.

The effect of lethal bacteremic shock on complement was

studied in the model developed during the last 2 years in this laboratory (Annual Reports 1960-1961, and 1961-1962). It is based on the discovery that most rabbits are naturally free of coliform bacilli so that E. coli of known serotype and pathogenicity can be introduced into the intestinal tract by feeding. Bacteremic shock due to E. coli of intestinal origin is then produced by two steps: (1) Intravenous injection of 3.0 mg./kg. nitrogen mustard and (2) rectal temperatures with probes* containing thermometric elements. At 90-100 hours after injection of nitrogen mustard, when granulocytopenia is most severe, animals develop high fever, overwhelming E. coli bacteremia and fatal shock. The origin of the bacteremia is traced to invasion of the rectal mucosa by rectal bacteria; from there the bacteria spread to the pelvic veins and induce pelvic thrombophlebitis.

In the present experiments coliform-free rabbits were fed 1.0 ml. of

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* Either thermistor probes (Glass enclosed thermometric element used with thermistor thermometer manufactured by E.H. Sargent Company) or soft internal (Esophageal-Rectal) probe covered with vinyl manufactured by Yellow Spring Instrument Company (See Figure 1, Annual Report 1960-1961).

an 18-hour culture of E. coli 0:113 in their drinking water for 3 days before administering nitrogen mustard and beginning rectal temperature probing. Blood was obtained for complement levels: (1) before feeding E. coli, (2) just before injecting nitrogen mustard, and (3) upon the appearance of a spike in fever. The blood for the complement determinations was placed in an ice bath immediately and the serum stored in the deep freeze at -70° C. until complement determinations were performed. The remaining serum was inactivated at 56° C. for 30 minutes and examined for antibody to endotoxin by the hemagglutination method.

In addition to the animals fed E. coli 0:113, 3 additional groups fed no E. coli, were examined for C' levels after neutropenia was produced by HN_2. The 3 additional groups were: (1) coliform-free rabbits; (2) coliform-free rabbits subjected to rectal probing; (3) rabbits with naturally heavy populations of E. coli in their stools. The following changes in complement levels were found:

Group	Animal #	Fever (Degrees F) When Bled for C'	Number Of Colonies Per ml. Blood	Change in C' Level (%)
Over-whelming Bacteremia Due to *E. coli* 0:113	1	4.5	Innumerable	+15.7
	2	4.3	480	+ 0
	3	4.3	2	+35.9
	4	5.0	600	+21.8
	5	4.4	3000	+11.3
	6	4.3	720	+ 8.9
	7	5.5	1320	-11
	8	5.3	22	+70
	9	5.0	11,880	-20
	10	3.8	16,200	+75
Over-whelming Bacteremia Due to *E. coli* Normally Present in Rabbits Own Stool	11	4.8	600	+20.4
	12	5.5	3000	0
	13	4.0	5	+12
	14	3.4	300	+30.6
	15	4.8	4500	+29
				- 8.4
Neutropenia 4 days after nitrogen mustard in coliform-free rabbits not fed *E. coli*; no rectal probing	16		ND	+20
	17		ND	+16
	18		ND	+33
	19		ND	0
	20		ND	0
Neutropenia 4 days after nitrogen mustard in coliform-free rabbits not fed *E. coli*, but given rectal probing	21		ND	+50
	22		ND	+90
	23		ND	+60
	24		ND	+89
	25		Sterile	60
	26		49*	46
	27		Sterile	69
	28		56*	183
	29		Sterile	39

ND = Not Determined

* = No *E. coli* — Only *Streptococcus viridans* and *Streptococcus fecalis*

The average change in C' level in 16 rabbits undergoing bacteremic shock was a small rise of 17.5%. In controls given the same manipulation without E. coli in stool and no E. coli bacteremia the average rise in C' was 78.4%. Interpolating from these control values, it can be concluded that the proctitis of rectal probing produced a marked rise in complement levels, from which a sharp fall develops when overwhelming bacteremia occurs. The mean fall in C' level would be: 78.4-17.5 = 60.9%. Experimental bacteremic shock due to E. coli, like endotoxin shock, is thus associated with a sharp drop in complement levels.

(b) Septic Arthritis of the Knee Due to E. coli.

Blood was removed by cardiac puncture from six normal rabbits in order to obtain baseline complement levels. Then 10^9 viable bacterial cells of an 18-hour culture of E. coli 0:113 was injected into the knee after the bacteria had been washed 3 times in sterile pyrogen-free saline. All rabbits developed severe arthritis with sublethal quantities of E. coli endotoxin circulating in the blood stream (4). Blood from the heart was obtained for complement levels at intervals of 1, 2, and 7 days after infection with the following results:

Rabbit	Interval After Inoculation of E. coli Into Knee (Days)	% Change in Complement Level
A	1	-34
	2	- 1.9
	7	+36.5
B	1	- 6.9
	2	-38.8
	7	+ 6.1
C	1	+22.6
	2	+19.4
	7	+13.5
D	1	+24.1
	2	+104.3
	7	+116.3
E	1	+26.8
	2	+104.9
	7	+104.1
F	1	-13.6
	2	0
	7	+40.6

It is apparent that all rabbits eventually developed a rise in C' levels, although half displayed an initial drop during the first 24-48 hours.

Summary of Observations on the Effects of Endotoxin on Complement

The inactivation of complement by an endotoxin varied with the bacterial species from which the endotoxin was derived and with the individual serum examined. Considerable differences in the depression of complement levels were observed among different endotoxins and different subjects. Each individual, however, exhibited its own unique pattern of complement inactivation with the battery of endotoxins

employed and the susceptibility to complement inactivation was stable from week to week. A good correlation was found between antibody (hemagglutinins) to endotoxin and complement inactivation by endotoxin; but lethality of endotoxin could not be related to its anticomplementary action *in vitro*. In experimental bacteremic shock from *E. coli*, however, the same drop in C' levels *in vivo* were observed as in animals given lethal doses of endotoxin. In sublethal joint infections with *E. coli*, the circulating levels of endotoxin produced transient falls in C' levels in half the animals and eventual elevations in circulating complement in all. These results strengthen the thesis that complement is involved in reactions with endotoxin *in vivo* and *in vitro* through a reaction with natural antibody; but the role of complement in mediating the lethal reaction in endotoxin shock requires further study to explain why C' depression *in vitro* and lethality of endotoxin could not be correlated with each other.

B. Protection Against Overwhelming Bacteremic Shock by Feeding E. coli.

Injection of sublethal doses of endotoxin not only stimulates antibodies to endotoxin, but also resistance to lethal doses. Last year we reported (Annual Report, 1962) that feeding E. coli also stimulated antibody formation to E. coli endotoxin and suggested that resistance to bacteremic shock due to E. coli might be increased by feeding that organism. The following experiments describe such an attempt to increase resistance to overwhelming bacteremia by feeding E. coli.

Twenty-four coliform-free rabbits were fed E. coli by placing 1.0 ml. of an 18-hour broth culture of E. coli 0:113 in their drinking water daily for 3 weeks before injection of nitrogen mustard (HN_2). A control group of 24 rabbits were fed E. coli in the same fashion but for only 3 days before receiving HN_2. Blood for antibody titers to E. coli endotoxin was taken by cardiac puncture before feeding was started and again just before HN_2 was injected. Forty-eight hours after each animal had received HN_2 (3 mg./kg.), the rectum was traumatized by taking continuous temperatures with the internal soft vinyl probe. The probe

was washed first with Zephiran solution, rinsed in tap water, dried with gauze and sterilized with 70% alcohol before temperatures were started each day. These precautions prevented transfer of intestinal bacteria from test animals to control animals. Fecal cultures showed that all rabbits had heavy populations in the stools when rectal temperatures were started. As soon as each animal developed fever, blood was drawn for culture and antibody titer. This fever usually took the form of a sudden spike 48 hours after rectal probing began. (See Figure 5, Annual Report, October, 1961).

The following tables compare the incidence of bacteremia, the height of fever, and the antibody titers in the two groups of rabbits.

Oral Immunization: E. coli O:113 Fed For 21 Days

Rabbit #	Maximum Fever (F)	Blood Culture Colonies E. coli/ml.	Reciprocal Hemagglut. Titer to E. coli O:113 Endotoxin				
			Before Feeding E. coli	After Feeding E. coli		Rise in Titer*	
				A	B	A	B
A-249	0	+	4	32		8	
B-249	3.8	12	ND	16		ND	
C-249	4.5	Sterile	2	8	8	4	4
D-249	2.8	Sterile	2	32	8	16	4
E-249	3.5	Innumerable	0	16	4	16	4
F-249	3.5	2	0	32	8	32	8
G-249	D	+	4	8		2	
H-249	3.1	Sterile	4	32	16	8	4
I-249	3.0	1	0	4	2	4	2
J-249	2.6	Sterile	0	16	8	16	8
K-249	D	Sterile	0	8		8	
L-249	D	Sterile	2	4		4	
A-253	2.5	Sterile	8	128	16	16	2
B-253	1.5	Sterile	4	16	8	4	2
C-253	1.4	Sterile	8	128	128	16	16
D-253	3.0	Sterile	2	256	256	128	128
E-253	1.5	Sterile	4	16	16	4	4
F-253	1.9	Sterile	2	32	32	16	16
M-253	2.2	Sterile	2	32	4	16	2
N-253	2.5	Sterile	16	512	128	32	8
O-253	3.1	Sterile	0	16	4	16	4
P-253	D	Sterile	0	32		32	
R-253	3.6	Sterile	0	8		8	

D = Died from Nitrogen Mustard Before Rectal Probing Began

\+ = Positive Culture of Heart Swab Post Mortem - No Plate Count

ND = No Determination Because of Accident

A = Just Before HN_2

B = 3-4 Days After HN_2 When Fever Spike Occurred

* = $\frac{\text{Titer Before Feeding E. coli}}{\text{Titer After Feeding E. coli}}$

Non-immunized Controls: E. coli O:113 Fed For 3 Days

Rabbit #	Maximum Fever Degrees (F)	Blood Culture Colonies E. coli/ml.	Reciprocal Hemagglut. Titer to E. coli O:113 Endotoxin	
			Before Feeding E. coli	After Feeding E. coli
			A	B
M-249	4.3	720	8	4
N-249	D	•	4	
O-249	D	Sterile	16	
P-249	5.5	1320	8	8
Q-249	D	Sterile	4	
R-249	5.3	22	2	4
S-249	D	Sterile	8	
T-249	5.0	11,880	2	0
U-249	3.8	16,200	2	2
V-249	4.1	Sterile	4	16
W-249	4.5	Sterile	4	2
X-249	D	Sterile	4	
G-253	3.0	Innumerable	2	2
H-253	3.5	Innumerable	4	2
I-253	4.2	Innumerable	8	16
J-253	3.9	Innumerable	4	4
K-253	D	Innumerable	2	
L-253	.4	Innumerable	2	2
S-253	3.5	•	0	
T-253	3.5	2040	0	0
U-253	D	Sterile	0	
V-253	D	•	2	
W-253	3.5	Innumerable	0	
X-253	D	Sterile		

D = Died from Nitrogen Mustard Before Rectal Probing Began

• = Positive Culture of Heart Swab Post Mortem - No Plate Count

ND = No Determination Because of Accident

A = Just Before HN_2

B = 3-4 Days After HN_2 When Fever Spike Occurred

The results of the two tables may be summarized as follows:

	Oral Immunization: Fed *E. coli* 21 Days Before HN_2	Non-immunized Controls: Fed *E. coli* 3 Days Before HN_2
Total Incidence of Bacteremia	20.8%	67%
*Corrected Incidence of Bacteremia	21 %	83%
Average Fever Maximum	2.8° F.	4.1° F.
Average Antibody Titer to *E. coli* O:113 Endotoxin Just Before HN_2	1:59	1:3
Average Antibody Titer to *E. coli* O:113 Endotoxin At Height of Fever (3-4 Days After HN_2)	1:40	1:5

*Incidence is corrected for animals that died from Nitrogen Mustard before rectal temperatures were taken.

These studies clearly demonstrate that bacteremic shock can be prevented by oral immunization. This oral immunization is accompanied by a sharp rise in specific antibody titer to *E. coli* endotoxin and would seem to offer protection by neutralizing endotoxin at the surface of the cell wall and thereby preventing sustained invasion of the blood stream. The protection by antibody against endotoxin cannot be the result of better phagocytosis by polymorphonuclear leukocytes because blood counts

showed that these cells had virtually disappeared from the blood of all animals in both groups on the fourth day after nitrogen mustard when fever spiked and blood cultures were performed. It is more likely that: (1) antibody reduced the ability of E. coli in the rectum to invade the traumatized pelvic veins and to establish thrombophlebitis, the essential preliminary to overwhelming bacteremia; (2) antibody increased the clearance of E. coli by the fixed macrophages of the RE system. The RE cells are resistant to HN_2, which does not impair clearance of E. coli or endotoxin from the blood (Annual Report, October 31, 1961).

These results also have three other important implications. First, they suggest for the first time a decisive importance of antibody to endotoxin in preventing infection. Previous studies with antibody have been concerned primarily with reducing toxicity of endotoxin. Second, they emphasize the importance of prior contact with Gram negative bacteria (low grade wound infection, intestinal colonization) in conferring resistance to bacteremic shock. Third, they suggest a possible role of prolonged antibiotic therapy in lowering resistance to bacteremia by eliminating from the bowel Gram negative bacteria that might otherwise

maintain immunity against blood stream invasion by antibiotic-resistant Gram negatives of the same bacterial species.

Summary of Oral Immunization Studies

Feeding E. coli 0:113 for 3 weeks stimulated antibody to the homologous endotoxin and protected animals with complete neutropenia against bacteremia arising from thrombophlebitis of pelvic veins. Antibody to endotoxin appeared to reduce the invasiveness of E. coli.

C. The Influence of Immunologic Paralysis Induced by Mitomycin C on Susceptibility to Endotoxin.

In order to gain more insight into immunologic factors responsible for resistance to endotoxin, an attempt was made to induce immunologic paralysis with Mitomycin C, an antagonist to the synthesis of DNA. Mitomycin is superior to nitrogen mustard and other alkylating agents for studies in mice and rats. These animals readily survive doses of Mitomycin C that produce the desired immunologic affect, but die quickly from comparable doses of HN_2 and related alkylating agents.

1. Influence of Mitomycin C on Lethality of Endotoxin.

Mitomycin C was dissolved in sterile saline and sublethal quantities given subcutaneously to groups of 35-40 mice in 3 different dosage schedules: (1) 0.1 mg. daily for 5 days; (2) 0.1 mg. for 1 dose only; (3) 0.2 mg. for 1 dose only. On the seventh day the treated mice, and corresponding numbers of untreated controls, were challenged by graded intravenous doses of endotoxin to determine the LD_{50}. In an additional group the LD_{50} was determined 24 hours after one subcutaneous injection of 0.1 mg. Mitomycin C. The dosage schedules of Mitomycin, the types of endotoxin,

their LD_{50}'s, and the change in susceptibility are summarized in the following table:

Experiment #	Dose Mitomycin (mg)	Interval Between 1st Dose Mitomycin And Challenge (Days)	Endotoxin	LD_{50} (mg)	Increased Susceptibility LD50 Controls LD_{50} Mit.-Tr. Mi.
1	0 (Control) 0.1 daily for 5 days	7	E. coli 0:113 E. coli 0:113	.155 .05	3.1
2	0 (Control) 0.1 for 1 dose	7	E. coli 0:113 E. coli 0:113	.141 .046	3.1
3	0 (Control) 0.1 for 1 dose 0.2 for 1 dose	7 7	E. coli 0:113 E. coli 0:113 E. coli 0:113	.158 .0312 .0312	5 5
4	0 (Control) 0.1 for 1 dose	7	S. flexneri S. flexneri	.125 .09	1.4
5	0 (Control) 0.1 for 1 dose 0.2 for 1 dose	7 7	S. flexneri S. flexneri S. flexneri	.177 .071 .0312	2.5 5.7
6	0 (Control) 0.1 for 1 dose 0.1 for 1 dose	1 7	E. coli 0:113 E. coli 0:113 E. coli 0:113	.155 .125 .084	1 1.8
7	0 (Control) 0.1 for 1 dose	7	S. typhimurium S. typhimurium	.250 .044	5.7

These results show that Mitomycin C lowers resistance to endotoxins of E. coli, Salmonella typhimurium, and Shigella flexneri; that a single dose is as effective as multiple doses of Mitomycin; and that more than 24 hours is required for lowering resistance to endotoxin. In view of the known antagonism of Mitomycin to DNA, and to protein synthesis, the results are consistent with suppressed production of a protective protein such as gamma globulin which has a half-life of 2 days in the mouse.

2. Effect of Mitomycin C on Antibody Formation to Endotoxin.

In order to determine whether Mitomycin C could depress antibody formation in mice this drug was given to mice immunized with Proteus mirabilis endotoxin. The endotoxin was prepared in this laboratory by the Boivin technique from bacteria grown in a synthetic medium containing only glucose, salts, and water (6).

Twenty-four Swiss-Webster mice were bled from the retroorbital capillary bed with a capillary pipette to obtain sera for precipitin and agglutination titers and then given an immunizing dose of .05 mg. Proteus endotoxin intraperitoneally. Twelve of the mice were then injected

subcutaneously with .05 mg. Mitomycin C daily and blood was obtained weekly thereafter for precipitin tests with Proteus endotoxin and agglutination tests with boiled Proteus bacilli.

The precipitin test was performed in capillary tubes measuring 1.5-2 mm. in diameter. A solution containing 1 mg. endotoxin/ml. saline was drawn into the tubes with serial 2-fold dilutions of sera pooled from 3 mice. The capillary tubes with serum and endotoxin were incubated at 37° C. for 2 hours, and overnight at 4° C. Then they were examined for precipitation and graded so that 1 mm. of precipitate = 1+. The agglutination test was done with cultures of Proteus mirabilis grown for 18 hours on plain agar washed off with saline and killed by placing the tubes in boiling water for 1 hour to destroy all antigens except the resistant "O" antigen (i.e. endotoxin). The suspension of dead bacteria was diluted with saline to a turbidity allowing 34% transmission at 610 mm. in the Beckman Jr. Spectrophotometer. One drop of the suspension was placed on glass slides with one drop of a serial dilution of pooled mouse serum and observed for macroscopic agglutination.

The following results were obtained:

PRECIPITIN TESTS

Pool #	Mitomycin	Week After Immunization	Serum Dilution 0	2	4	8
1	Yes	0	0	0	0	0
		1	1+	±	0	0
		2	1+	1+	0	0
		3	1+	1+	±	0
2	Yes	0	0	0	0	0
		1	1+	±	0	0
		2	1+	0	0	0
		3	1+	±	0	0
3	Yes	0	0	0	0	0
		1	1+	±	0	0
		2	1+	0	0	0
		3	3+	2+	1+	±
4	Yes	0	0	0	0	0
		1	1+	0	0	0
		2	1+	0	0	0
		3	±	±	0	0
5	No	0	0	0	0	0
		1	4+	2+	1+	0
		2	4+	2+	2+	±
		3	3+	2+	1+	0
6	No	0	0	0	0	0
		1	3+	2+	1+	0
		2	3+	2+	1+	0
		3	3+	2+	1+	0
7	No	0	0	0	0	0
		1	3+	2+	1+	
		2	3+	2+	1+	±
		3	3+	2+	1+	1+
8	No	0	0	0	0	0
		1	3+	2+	1+	0
		2	3+	2+	1+	0
		3	3+	2+	2+	1+

AGGLUTINATION TESTS

Pool #	Mitomycin	Reciprocal Agglutinin Titer Before Immunization	1 Week	2 Weeks
1	Yes	0	10	5
2	Yes	ND	5	5
3	Yes	0	5	0
4	Yes	0	0	5
1	No	ND	40	80
2	No	0	20	20
3	No	0	10	5
4	No	0	40	40

ND = No Determination

These precipitin and agglutination tests both demonstrate clearly that Mitomycin C blocks the formation of antibody to endotoxin ("O" antigen). The major observable gross effect in these mice given Mitomycin in this experiment was marked atrophy of the spleen at 2-3 weeks.

When the dose of Mitomycin was cut in half (.025 mg.) only slight inhibition of antibody formation was noted.

3. Distribution of Mitomycin in Viscera.

In order to determine whether Mitomycin localized in the reticuloendothelial organs responsible for inactivation of endotoxin, assays for Mitomycin were performed with various tissues at 2 and 4 hours after injection of Mitomycin.

Four Swiss-Webster mice, weighing 20 grams, were inoculated intravenously with 0.5 ml. of a 3 hour culture of Proteus vulgaris (Strain #6731). Immediately afterwards, 2 of these mice each received subcutaneous injections of .25 mg. Mitomycin C in the shoulder. The other 2 mice served as controls. Two hours later 1 mouse inoculated with Mitomycin and 1 control were exsanguinated through the retroorbital plexus and the liver, spleen, and kidneys removed. Four hours later the remaining 2 mice were treated similarly. The tissues were minced, placed in 5 ml. trypticase soy broth and allowed to stand at 4° C. overnight. The next morning the suspensions were centrifuged at 2000 rpm for 5 minutes and the supernates transferred to sterile test tubes. These tubes were incubated at 37° C. and growth measured turbidimetrically every hour in the Coleman Jr. Spectrophotometer at 610 mm. The amount of Mitomycin C in tissues was determined by measuring the reduction of turbidity from bacterial growth (increased light transmission). The assay* was then repeated but the Mitomycin

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* This assay for Mitomycin in vivo was developed in this laboratory after preliminary studies showed that Mitomycin would not diffuse from tissue extracts placed in oxford cups on plates seeded with bacteria or into supernatant broth subsequently inoculated with bacteria. The present assay measured Mitomycin's action against Proteus in vivo.

was given 1 hour after inoculation of P. vulgaris. In addition to the 2 mice receiving .25 mg. Mitomycin C, 2 others received .10 mg. Mitomycin C. The following results were obtained:

Tissue Supernate	Dose Mitomycin (mg.)	Hours After Inoculation of P. vulgaris	Hours After Mitomycin	Growth Turbidity After Incubation at 37° C. (% Transmission) Initial	11 Hours	% Inhibition Of Growth By Mitomycin C*
Blood	.25	2	2	73	66	30
Kidneys				71	67	35
Liver				64	46	36
Spleen				72	66	44
Blood	0	2	2	74	37	
Kidneys				67	28	
Liver				65	11	
Spleen				74	24	
Blood	.25	4	4	72	47	5
Kidneys				72	46	5
Liver				46	37	43
Spleen				70	60	34
Blood	0	4	4	70	40	
Kidneys				67	36	
Liver				59	7	
Spleen				69	25	
Blood	.10	3	2	63	52	21
Kidneys				60	55	18
Liver				57	36	19
Spleen				65	45	24
Blood	.25	3	2	67	55	20
Kidneys				60	59	22
Liver				59	53	34
Spleen				60	42	26
Blood	0	3	2	59	27	
Kidneys				53	30	
Liver				46	6	
Spleen				60	16	
Blood	.10	5	4	63	44	6
Kidneys				60	56	12
Liver				61	55	35
Spleen				64	49	28
Blood	.25	5	4	69	66	22
Kidneys				55	51	12
Liver				42	32	31
Spleen				67	58	34
Blood	0	3	4	64	39	
Kidneys	(Control)			65	49	
Liver				54	13	
Spleen				64	21	

* % Inhibition of Growth = Change in turbidity of test cultures minus change in turbidity of control cultures (0 Mitomycin)

There was marked inhibition of bacterial growth by Mitomycin (through DNA inhibition in Proteus) in supernates of the liver and spleen (especially apparent at 4 hours after Mitomycin had been excreted from the blood of several animals). This demonstrates heavy localization of Mitomycin in liver and spleen, the organs primarily involved in inactivation of endotoxin. Increased susceptibility to endotoxin after 1 injection of Mitomycin could thus be explained by inhibition of DNA synthesis in liver and spleen with suppression of normal mitosis of phagocytic and antibody producing cells. Normal DNA synthesis thus appears to be essential for full resistance to endotoxin.

4. Effects of Mitomycin C on Circulating Leukocytes and Lymphatic Tissues.

Mice were given daily subcutaneous injection of Mitomycin C in doses of either 0.1 mg., 0.5 mg., 0.25 mg., or .01 mg.; and blood obtained weekly for WBC and differential counts from the retroorbital plexus with the following results:

Weeks After Mitomycin:

Mouse #	Dose Mitomycin (mg.)	0 Total WBC	0 Total PMN	1 Total WBC	1 Total PMN	2 Total WBC	2 Total PMN	3 Total WBC	3 Total PMN
1	.100	4400	1496	5400	1404	Died			
2	.100	5850	1462	6400	2112	900	90		
3	.050			14250	2420	2250	698	1125	180
4	.050			7600	2660	1400	210		
5	.025			8100	2430	5300	1961	2650	371
6	.025			8550	4983	5350	1605	1125	371
7	.010					5100	1122	1800	450
8	.010					5825	1250	2750	798

The leukopenia was accompanied by a comparable thrombocytopenia and atrophy of the spleen, cecum, and other lymphatic tissues. It should be noted that no leukopenia developed in the peripheral blood until after the first week of injections.

Another 15 mice were given 5 daily injections of .100 mg. Mitomycin C subcutaneously and sacrificed 3 days after the last dose. The spleen weights ranged from .02-.03 mg. and the average was .024 mg. The spleens of normal controls weighed .135-.185 mg. with an average of .156 mg. The cecums of mice treated with Mitomycin were also markedly atrophic but other tissues appeared normal and the total body weights of

treated mice were not reduced. Spleens began to regain their weight about 11 days after the last of 5 doses of .100 mg. of Mitomycin C.

Because one injection of 0.1 mg. Mitomycin lowered resistance to endotoxin one week later, a study was made of mice receiving this dosage of Mitomycin. Six Swiss-Webster mice, age 3 months, and weighing 30-39 grams were given an injection of 0.1 mg. Mitomycin C subcutaneously and blood counts taken from the retroorbital plexus before, and 1 week after the injection. No significant change was found at one week in blood hemoglobin levels and body weight. Spleens of mice with Mitomycin C did not weigh less than the controls. The only change occurred in the WBC of four mice as follows:

	Total WBC		% Polymorphonuclears	
Mouse #	Before Mitomycin	1 Wk. After Mit.	Before Mitomycin	1 Wk. After Mit.
7	11,300	3164	4100	205
9	15,000	2550	6875	1581
11	10,000	3600	6425	2249
12	10,750	1828	5000	1100

These leukocyte changes were not considered great enough to account for the increased susceptibility of mice to endotoxin because the counts after Mitomycin were not below the levels seen in normal control animals.

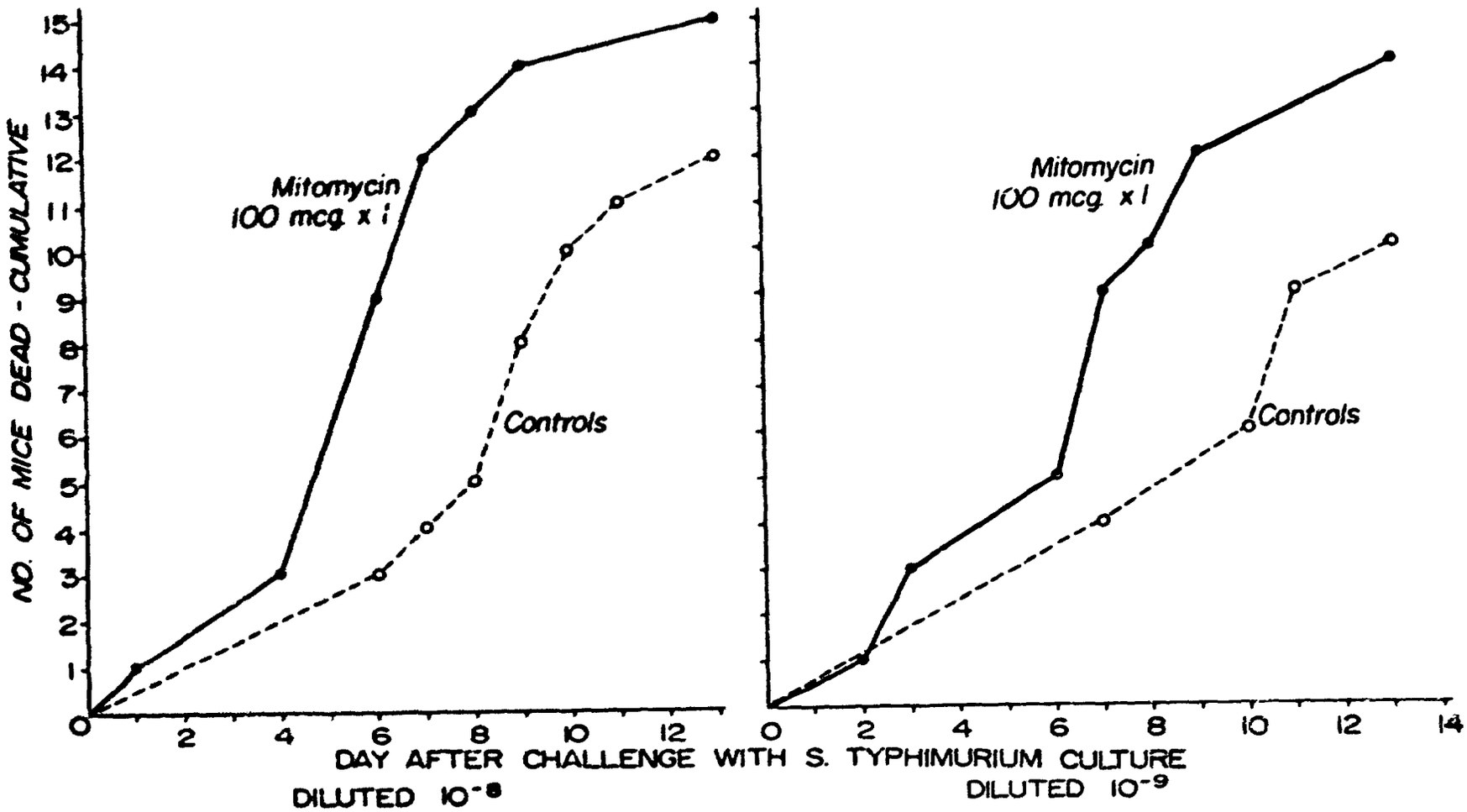
4. The Effect of Mitomycin C on Resistance to Lethal Salmonella Infection.

In view of the lowered resistance to Salmonella endotoxin after the injection of 0.1 mg. Mitomycin, a study was made to determine whether Mitomycin similarly lowered resistance to Salmonella infection. If this were the case, it would substantiate the impression (gained from studies with bacteremic shock in Section B) that resistance to endotoxin parallels resistance to infection by Gram negative bacteria.

Preliminary studies had shown that 10^{-8} and 10^{-9} dilutions of 18-hour trypticase soy broth cultures of Salmonella typhimurium were lethal for mice approximately 2 weeks after intraperitoneal inoculation. The following experiment was conducted to determine if 0.1 mg. Mitomycin would accelerate the death rate from these small numbers of S. typhimurium. A group of thirty 8-week-old white Swiss mice were given 0.1 mg. Mitomycin subcutaneously and one week later were challenged with S. typhimurium. Fifteen of these mice received 0.5 ml. of a 10^{-8} dilution and 15 received 0.5 ml. of a 10^{-9} dilution of an 18-hour broth culture of S. typhimurium. An equal number of control mice received each of the 2 doses of Salmonella and the mortality rates compared with those in the mice that had received

FIGURE 4

EFFECT OF MITOMYCIN ON SUSCEPTIBILITY TO LETHAL SALMONELLA BACTEREMIA



0.1 mg. Mitomycin 7 days previously.

Figure 4 shows that mice treated with Mitomycin died faster than controls given either 10^{-8} or 10^{-9} dilutions of Salmonella cultures. This increased susceptibility to S. typhimurium infection parallels the increased susceptibility to S. typhimurium endotoxin induced by Mitomycin C.

Summary of Observations with Mitomycin C

The DNA antagonist, Mitomycin C, which inhibited antibody formation to endotoxin, lowered resistance to infection by Gram negative bacteria and to the lethal effects of their endotoxin.

D. A Comparison of the Toxic and Antigenic Properties of Endotoxin Purified By Density Gradient Zone Ultracentrifugation.

The parallelism between the immunologic and toxic properties of endotoxin implies a single molecular configuration responsible for both properties. In order to obtain further information on the association between toxicity and antigenicity, endotoxin was subjected to ultracentrifugation in a sucrose gradient. If the endotoxin preparation were a mixture of two different substances, one a toxin and the other an antigen, they might be separable in a density gradient.

In order to detect the distribution of the endotoxin in the gradient it was labelled internally with d-glucose labelled uniformly with C-14. For this purpose E. coli 0:113 was selected as the source of endotoxin because it can be grown in a synthetic medium providing glucose as the sole source of carbon. The endotoxin was extracted with trichloroacetic acid by the standard method in this laboratory, and possessed radioactivity equivalent to 6000 counts per minute in the Packard Tricarb Liquid Scintillation Counter. The LD_{50} in 8-week old white mice was .217 mg. The LD_{50} of non-labelled endotoxin prepared simultaneously was .218 mg.

Sucrose gradients from 1 M to .2 M were prepared by adding pyrogen-free distilled water to a mixing chamber containing a 1 M pyrogen-free sucrose solution in pyrogen-free distilled water with continuous stirring by a magnetic stirrer. The sucrose solution of decreasing molarity was carefully layered into a centrifuge tube by means of a polyvinyl tube to a volume of approximately 28 ml. The sample containing 1 mg. of endotoxin dissolved in 1 ml. 5% glucose was layered on the top by means of a syringe.

Ultracentrifugation was carried out in a Spinco Model L ultracentrifuge using a SW 25.1 swinging bucket rotor for 17 hours at 25,000 rpm, with the running temperature +5° C.

After centrifugation the bottom of the centrifuge tube was perforated and fractions of 1 ml. were collected, so that the densest material appeared in tube 1 and the least dense in tube 29. Absorbance was measured at 254 mu and radioactivity was determined by suspending aliquots of the fractions in thixotropic gel and counting in the Packard Tricarb Liquid Scintillation Counter.

The toxicity and antigenicity of each of the 29 fractions collected from the sucrose gradient were determined by inoculating rabbits intravenously with serial 10-fold dilutions. The minimal pyrogenic dose of each fraction

was assayed by the method of Keene et al. (5), and the pyrogenic activity per microgram of material was then determined from the radioactivity on the basis of 6000 cts/minute/mg.

The minimal antigenic dose was determined by calculating the highest dilution that produced a 4-fold rise in serum titer of hemagglutinating antibodies against red cells sensitized with endotoxin. The minimal antigenic dose was assayed in rabbits at the same time that the minimal pyrogenic dose was measured. Each rabbit thus received one injection of the material from the sucrose gradient; and blood for antibody was drawn before and 1 week after the pyrogen test. The rises in titer at 1 week were plotted against the dilution of test material on graph paper in order to determine the highest dilution that produced a 4-fold rise.

The results are shown in Figure 5. Radioactivity and absorbancy at 254 mu give good agreement for distribution through the gradient. The bulk of the material was of low density, relatively non-toxic (tubes 18-29), and constituted impurities from which the active purified fraction was separated by virtue of its greater density.

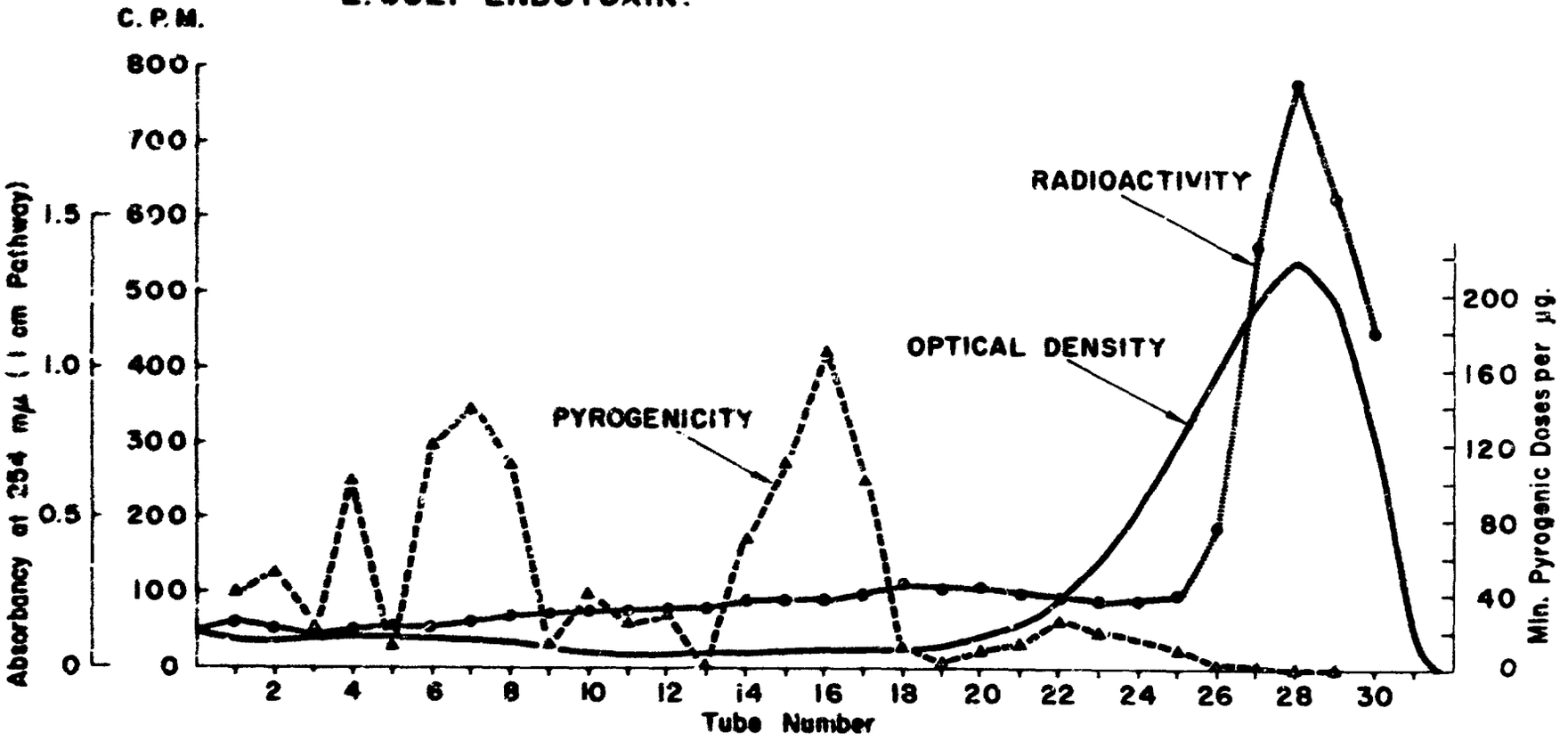
Toxicity (pyrogenicity) was present primarily in the denser fractions, at the middle and lower portions of the gradient (Figure 5). Antigenicity was also concentrated in the denser material in the lower section of the gradient (tubes 1-16) as the following table illustrates:

Tube	Minimal Antigenic Doses Per Microgram	Tube	Minimal Antigenic Doses Per Microgram
1	100	17	6
2	600	18	0
5	100	19	60
6	100	20	5
14	0	22	120
15	100	25	5
16	300	26	0.3
		27	10
		28	0
		29	1000

A notable exception is the presence in tube 29 of a very light non-toxic material possessing considerable antigenicity. This corresponds to the non-toxic antigen previously isolated in this laboratory by treatment with $LiAlH_4$ (6), to the lightweight antigenic, but non-toxic preparation observed in immunodiffusion studies by Chedid and Skarnes (7), and to the non-toxic antigen extracted by Ribi with Dioxan (8).

FIGURE 5

SUCROSE GRADIENT DISTRIBUTION OF RADIOACTIVITY OPTICAL DENSITY AND PYROGENICITY OF C-14 LABELED E. COLI ENDOTOXIN.



This technique offers considerable promise for obtaining a purified endotoxin because the antigenic and toxic (pyrogenic) activity in the most active portion of the gradient is 50-100 times greater than that in the parent material.

Summary of Experiments With the Sucrose Gradient

Purified fractions of endotoxin with high toxic and antigenic activity were concentrated in the dense portions of the gradient and separated from the bulk of the less dense material. These studies further illustrate the parallelism of the toxic and antigenic properties of endotoxin and imply that a single molecular configuration is responsible for both properties. This new evidence that toxicity and antigenicity go hand in hand is consistent with the view that endotoxic shock is a form of anaphylactic shock.

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